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13. ABSTRACT ***(Maximum 200 Words)***

HGF (also known as scatter factor), and its receptor Met, are over-expressed in invasive human breast cancer. In addition, increased HGF expression and sustained activation of Met have been shown to be important steps in the development of metastatic breast cancer. Therefore, HGF-Met binding is a potential target for anti-cancer antagonists in the treatment of breast cancer metastasis. We have developed an HGF-Met binding assay where Met is immobilized on a plastic plate, followed by the addition of HGF and subsequent detection of HGF binding. Addition of varying compounds/peptides can be monitored for induction of increased or decreased HGF-Met binding. We are using a new technology, known as "phage display", to isolate short peptides which bind specifically to HGF or Met and block growth factor function. Additionally, we have shown that certain divalent cations (e.g., Cu^{2+}) can inhibit HGF-Met binding. Once antagonistic peptides/compounds have been identified, their putative effect on cell functions such as Met activation, cell motility and invasion will be assessed. This approach could lead to the development of novel inhibitors of HGF function in carcinoma cells, and new strategies for improved treatment of breast cancer, perhaps in combination with other anti-cancer agents.

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PREFACE:

As noted in the covering letter, this PhD trainee programme has been modified. The original PI (Jin Gui) withdrew from the programme in May 2000 for academic and personal reasons. After an extensive recruitment effort, a new PhD student, Ted Wright, was hired in May 2001 to continue this project. Mr. Wright completed his under the supervision of Dr. M. Koschinsky (Dept. Biochemistry, Queen's University) on the role of kringle domains in the assembly of lipoprotein (a). His formal training in protein biochemistry and kringle domain biology provides an ideal background for this project. Dr. Bruce Elliott, Professor, Dept. Pathology, continues to be the supervisor of this programme. The duration of the award has been extended to August 2003 (with no additional money). A revised statement of work was submitted, and approved by the USAMRMC (See App. I).

INTRODUCTION:

Breast cancer is a major concern in North America, with 1 in every 8 women developing breast cancer and 1 in 3 of these women dying from metastasis. Dr. Elliott's laboratory and others have previously demonstrated elevated HGF and Met expression in regions of invasive human breast carcinomas (1,2), suggesting a role of autocrine HGF loops in invasive breast cancer. In addition, increased HGF expression and sustained activation of Met have been shown to be important steps in the development of metastatic breast cancer (3,4). Therefore, HGF-Met binding is a potential target for anti-cancer antagonists in the treatment of breast cancer metastasis.

Met is composed of two components (chains), known as α and β, with the α chain embedded in the membrane of epithelial cells and the β chain exposed at the surface of the cells, free to interact with HGF. HGF is produced as an inactive protein which is then processed to an active form, also composed of α and β chains. The α chain of HGF is known to interact with the α chain of Met. The HGF-α chain is composed of an N-terminus followed by four consecutive structures known as kringles, designated K1, K2 , K3 and K4. HGF interaction with Met is mediated mainly by K1 and somewhat by K2 (5). We are therefore developing small peptide mimetics that inhibit the interaction of K1 and K2 of HGF with Met (Obj. 2).

The tumor microenvironment may be important in regulating HGF expression, processing and degradation in breast carcinoma cells. In addition, production of naturally occurring isoforms, such as NK2 (Mr-30 kDa), or proteolytic degradation may have agonistic or antagonistic effects on Met activation in carcinoma cells. We are using a human breast carcinoma cell line (MCF10A1T3B), which over-expresses the *Ha-ras* oncogene (6,7), to study maturation and degradation of HGF isoforms in breast cancer (Obj. 1).

BODY:

Hypothesis and Objectives:

Our **hypothesis** is that HGF interaction with, and subsequent activation of Met, is a major step in transition of epithelial cells to a malignant phenotype. Hence, disruption of this interaction may play an important role in inhibiting these functions.

Two overall **objectives** are proposed:

1) To identify, and assess the structural properties, of native HGF, HGF degradation products and isoforms secreted by breast carcinoma cell lines and tissues; then to further determine the effects of putative HGF degradation products and isoforms on Met activation and function in breast epithelial and carcinoma cells, and

2) To design high affinity peptides that either inhibit or promote the interaction between HGF and Met and analysis of these peptides on the modulation of HGF/Met function.

Objective 1: Over-expression of *Ha-Ras* in a human breast epithelial cell line MCF10A1 (referred to as MCF10A1T3B cells) causes increased filopodia extensions (Figure 1), cell scattering, and tumorigenesis in nude mouse xenografts (6,7). Dr. Elliott's laboratory has shown increased expression of mature HGF in conditioned media (CM) from MCF10A1T3B cells, as well as lower M_r HGF immmunoreactive bands (56 kDa and 32 kDa) (Figure 2). The smaller M_r HGF form in MCF10A1T3B CM may be related to the HGF NK2 isoform, which has a M_r of approximately 32 kDa (8,9); the larger form could represent an intermediate stage of processing. Similar low M_r forms of HGF were produced by degradation from full-length rHGF, following incubation with MCF10A1T3B cells (data not shown). To determine whether proteolytic processing of HGF is linked to the EMT phenotype of MCF10A1T3B cells, we will test whether neutralizing anti-HGF IgG can inhibit Met activation and scattering of MCF10A1T3B cells. Conversely, we will test whether CM from MCF10A1T3B cells stimulates scattering (or Met activation) of MCF10A1 cells, and whether HGF is involved. Further experiments will be performed to:

a) identify putative HGF forms using western blot analysis with antibodies specific for NK1 (from Elliott lab.) or NK2 (10) domains;
b) test the activity of lower M_r HGF forms, purified from MCF10A1T3B cells using ionic exchange chromatography, in Met activation and functional assays;
c) using mass spectroscopy, characterize and sequence peptide fragments prepared from rHGF following degradation by MCF10A1T3B CM or cell membrane lysates, and
d) identify proteolytic activity in MCF10A1T3B CM or cell membrane extracts responsible for generating the putative HGF forms, using specific inhibitors(11,12) of cysteine (E64 (13)), serine (benzamidine, DIFP (14)), and metalloproteinases (BB-94 (15)), and zymography with casein and gelatin (broad range) substrates. Protease inhibitors will also be used to assess the role of proteolysis in HGF/Met function (16).

Objective 2:

a) Develop an efficient ELISA screening assay for HGF/Met binding:

As a first step, we have developed an ELISA assay to study HGF/Met binding. The assay involves chimeric Met-IgG fusion protein or HGF as substrate immobilized on plastic, followed by the addition of HGF or Met, and subsequent detection binding to the appropriate substrate. An overview of the ELISA strategies used is shown in Figure 3. The results showed a linear increase in HGF binding to immobilized Met-IgG, compared to a fibronectin control, which showed no significant binding (Figure 4). These results indicate that an ELISA assay can be used to detect HGF/Met binding in a semi-quantitative manner.

b) **Screen for candidate compounds**:

We have previously shown that HGF is a Cu^{2+}-binding protein (17). This property has been useful in developing a one-step purification process for HGF from conditioned media or cell lysates. The Cu^{2+}-binding characteristics of HGF raise the possibility that Cu^{2+} may modulate HGF/Met binding and function. We therefore examined the effect of Cu^{2+} on HGF/Met binding in the ELISA assay. To avoid nonspecific effects of Cu^{2+}, Met was directly coated onto the plastic plate, and HGF was added with, or without Cu^{2+}. Excess HGF and Cu^{2+} was removed and residual HGF binding was detected using anti-HGF IgG. The results showed that 2.5 mM to 20 mM Cu^{2+} completely inhibited HGF binding to Met-IgG, whereas 1 mM Cu^{2+} partially inhibited binding (Figure 5). Interestingly, Cu^{2+} had no effect on binding of HGF previously incubated with immobilized Met (Figure 6). Together, these findings indicate that Cu^{2+} is unlikely to interfere with HGF already bound to Met, although it may prevent or reduce binding to unoccupied Met.

Considering the role of lysine residues in kringle domain protein-protein interactions (18), we examined whether lysine was involved in binding of HGF to Met. The results showed that pre-incubation of HGF with the lysine analogue, ε-ACA, had no effect on binding to Met (Figure 7). A similar approach will be used to assess the role of other charged residues, eg histidine in Cu^{2+} binding, by testing inhibition with DEPC.

As a first step to examine the effect of Cu^{2+} on HGF/Met function, we determined whether addition of 0.5 M Cu^{2+} could inhibit HGF-induced cell scattering of MDCK cells. The results showed a strong reduction in HGF-induced scattering following treatment with 0.5 M Cu^{2+}, compared to a control group stimulated with HGF alone (Figure 8A-8D). Cells treated with HGF and 0.5 M Cu^{2+} showed a more spread-out morphology, compared to untreated cells. Nuclei of cells treated with Cu^{2+} remained intact as assessed by DAPI staining (data not shown), indicating absence of apoptotic or necrotic cell death. These results show that Cu^{2+} blocks the scattering function of HGF in MDCK cells. Experiments are in progress to test the effect of Cu^{2+} on HGF-induced Met kinase activity and other functions.

Future directions include: a) screening other candidate divalent cations (eg Zn^{2+} and Fe^{2+}), and b) determining how Cu^{2+} inhibits HGF and Met binding, using better resolution techniques such as circular dichroism, surface plasma resonance (SPR), and differential scanning colorimetry. These approaches can be used to assess association constants, pH curves, and energy transfer of the binding reaction. Ultimately, our plan is to perform crystallographic studies of Cu^{2+}/HGF complexes.

c) Set-up phage display system and screen peptides:

We are using a new technology, known as "phage display" (18), to isolate short peptide mimetics, which bind specifically to HGF or Met and block growth factor function (Figure 9). Once antagonistic peptides/compounds have been identified, their putative effect on cell functions such as Met activation, cell motility and invasion, will be assessed. We are proceeding as described in the attached Statement of Work.

Key Research Accomplishments:

- MCF10A1T3B breast carcinoma cells express degradation products of HGF, which once isolated can be used for competitive assays of HGF-Met binding.
- An ELISA system can be used initially to detect HGF and Met binding, and can assess inhibition of binding.
- Cu^{2+} appears to inhibit HGF-Met binding, perhaps due to changes in HGF conformation.

Reportable outcomes:

- Canadian Breast Cancer Research Initiative IDEA grant applied for and awarded to BE, 1999-2000 (C$46,000 over 2 yr). See Abstract attached.
- PhD candidate Ted Wright began work on this project in May 2001.

Conclusions:

Results reported in this period have focused on Objectives 2a and 2b. Experiments to examine HGF isoform differences expressed in normal and malignant breast epithelial cells and to examine possible functions of these isoforms (Objective 1) are in progress and will be described in the next reporting period.

The ELISA assay described in this report represents an important first step in our study of HGF/Met binding. This assay has been optimized to detect HGF/Met binding in a semi-quantitative manner. However, approaches to develop antibody-independent assays that will provide more biophysicial infomation about the HGF/Met interaction are in progress. Our long term goal is to determine the 3-D structure of Met and the Met/HGF complex by crystallography (collaboration with Dr. Z. Jia). This information will be used in developing small molecules with

enhanced binding affinity to Met or HGF, and capable of blocking Met activation by native HGF.

Our novel observation that Cu^{2+} can inhibit binding of HGF to Met raises the intriguing possibility that divalent cationic metal binding may affect the conformation of the HGF molecule, thereby modulating its Met binding properties. We intend to examine Cu^{2+} binding effects as a parallel approach to the design of small molecule inhibitors of HGF/Met interactions.

This study will provide new information regarding HGF regulation in breast cancer. It could lead to the development of novel inhibitors of HGF function in carcinoma cells, and new strategies for improved treatment of breast cancer, perhaps in combination with other anti-cancer agents.

Reference List

1. Tuck, A.B., Park, M., Sterns, E.E., Boag, A., and Elliott, B.E. Coexpression of hepatocyte growth factor and receptor (Met) in human breast carcinoma. Am. J. Path., *148*: 225-232, 1996.

2. Ghoussoub, R.A.D., Dillon, D.A., D'Aquila, T., Rimm, E.B., Fearon, E.R., and Rimm, D.L. Expression of c-Met is a strong independent prognostic factor in breast cancer. Cancer, *82*: 1513-1520, 1998.

3. Takayama, H., LaRochelle, W.J., Sharp, R., Otsuka, T., Kriebel, P., Anver, M., Aaronson, S.A., and Merlino, G. Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. Proc. Natl. Acad. Sci.USA, *94*: 701-706, 1997.

4. Nagai, S., Kitani, S., Hirai, K., Takaishi, T., Nakajima, K., Kihara, H., Nonomura, Y., Ito, K., and Morita, Y. Pharmacological study of stem-cell-factor-induced mast cell histamine release with kinase inhibitors. Biochem. Biophys. Res. Commun., *208*: 576-581, 1995.

5. Lokker, N.A., Presta, L.G., and Godowski, P.J. Mutational analysis and molecular modeling of the N-terminal kringle-containing domain of hepatocyte growth factor identifies amino acid side chains important for interaction with the c-Met receptor. Protein Engineering, *7*: 895-903, 1994.

6. Santner, S.J., Dawson, P.J., Tait, L., Soule, H.D., Eliason, J., Mohamed, A.N., Wolman, S.R., Heppner, G.H., and Miller, F.R. Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. Breast Cancer Res Treat, *65*: 101-110, 2001.

7. Giunciuglio, D., Culty, M., Fassina, G., Masiello, L., Melchiori, A., Paglialunga, G., Arand, G., Ciardiello, F., Basolo, F., and Thompson, E.W. Invasive phenotype of MCF10A cells overexpressing c-Ha-ras and c-erbB-2 oncogenes. Int. J. Cancer, *63*: 815-822, 1995.

8. Montesano, R., Soriano, J., Malinda, K., Ponce, M., Bafico, A., Kleinman, H., Bottaro, D., and Aaronson, S. Differential effects of hepatocyte growth factor isoforms on epithelial and endothelial tubulogenesis. Cell Growth Differ., *9*: 355-365, 1998.

9. Miyazawa, K., Kitamura, A., Naka, D., and Kitamura, N. An alternatively processed mRNA generated from human hepatocyte growth factor gene. Eur. J. Biochem., *197*: 15-22, 1991.

10. Fehlner-Gardiner, C.C., Cao, H., Jackson-Boeters, L., Nakamura, T., Elliott, B.E., Uniyal, S., and Chan, B.M. Characterization of a functional relationship between hepatocyte growth factor and mouse bone marrow-derived mast cells. Differentiation, *65*: 27-42, 1999.

11. Overall, C.M. Matrix metalloproteinase substrate binding domains, modules and exosites. Overview and experimental strategies. Methods Mol. Biol., *151*: 79-120, 1901.

12. Zeslawska, E., Schweinitz, A., Karcher, A., Sondermann, P., Sperl, S., Sturzebecher, J., and Jacob, U. Crystals of the urokinase type plasminogen activator variant beta(c)-uPAin complex with small molecule inhibitors open the way towards structure-based drug design. J. Mol. Biol., *301*: 465-475, 2000.

13. Stonelake, P.S., Jones, C.E., Neoptolemos, J.P., and Baker, P.R. Proteinase inhibitors reduce basement membrane degradation by human breast cancer cell lines. Br. J. Cancer, *75*: 951-959, 1997.

14. Forney, J.R., Yang, S., and Healey, M.C. Protease activity associated with excystation of Cryptosporidium parvum oocysts. J. Parasitol., *82*: 889-892, 1996.

15. Lee, J., Weber, M., Mejia, S., Bone, E., Watson, P., and Orr, W. A matrix metalloproteinase inhibitor, batimastat, retards the development of osteolytic bone metastases by MDA-MB-231 human breast cancer cells in Balb C nu/nu mice. Eur. J. Cancer, *37*: 106-113, 2001.

16. Leung, D., Abbenante, G., and Fairlie, D.P. Protease inhibitors: current status and future prospects. J. Med. Chem., *43*: 305-341, 2000.

17. Rahimi, N., Etchells, S., and Elliott, B. Hepatocyte growth factor (HGF) is a copper-binding protein: a facile probe for purification of HGF by immobilized Cu(II)- affinity chromatography. Protein Express. Purif., *7*: 329-333, 1996.

18. Gherardi, E., Hartmann, G., Hepple, J., Chirgadze, D., Srinivasan, N., and Blundell, T. Domain structure of hepatocyte growth factor/scatter factor (HGF/SF). Ciba Found. Symp., *212*: 84-93, 1997.

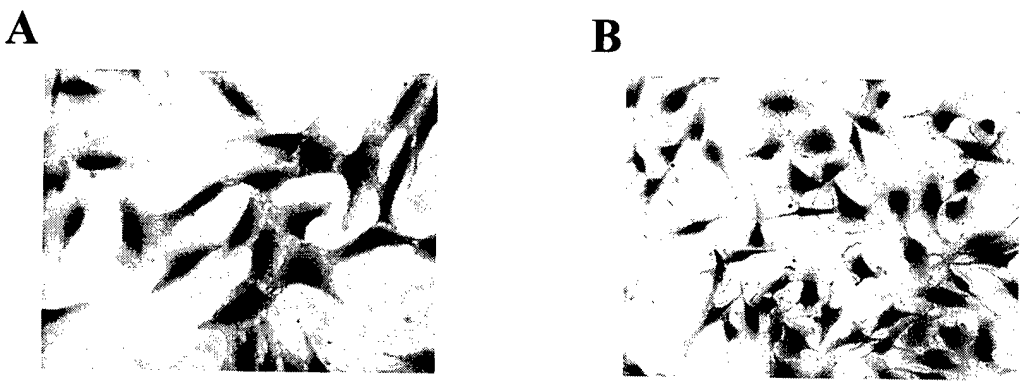

Figure 1. Transforming effect of *Ha-Ras* on morphology of human breast epithelial cells: A) A normal human breast epithelial cell line MCF10A1, showed cell spreading with cell-cell contacts. **B)** *Ha-Ras*-transfected MCF10A1 cells (designated MCF10A1T3B) showed increased filopodia and lamellipodia formation and cell scattering. (200x magnification)

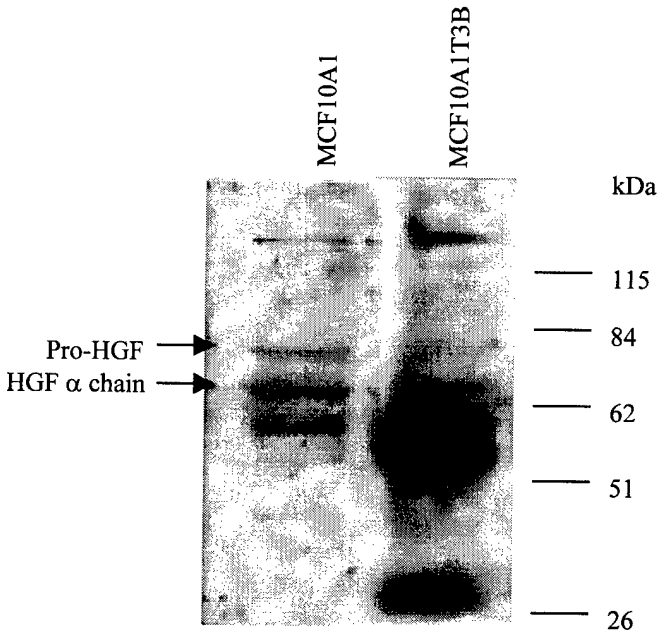

Figure 2. HGF expression status of MCF10A1 breast epithelial cells: CM from MCF10A1 and MCF10A1T3B cell lines were purified using Cu (II) affinity chromatography. The eluted fractions were then assessed for HGF expression by subjection to reduced 9% SDS-PAGE and blotting with rabbit anti-human HGF IgG. MCF10A1T3B CM showed increased presence of HGF (α chain), as well as two lower Mr HGF immunoreactive bands (56 kDa and 32 kDa).

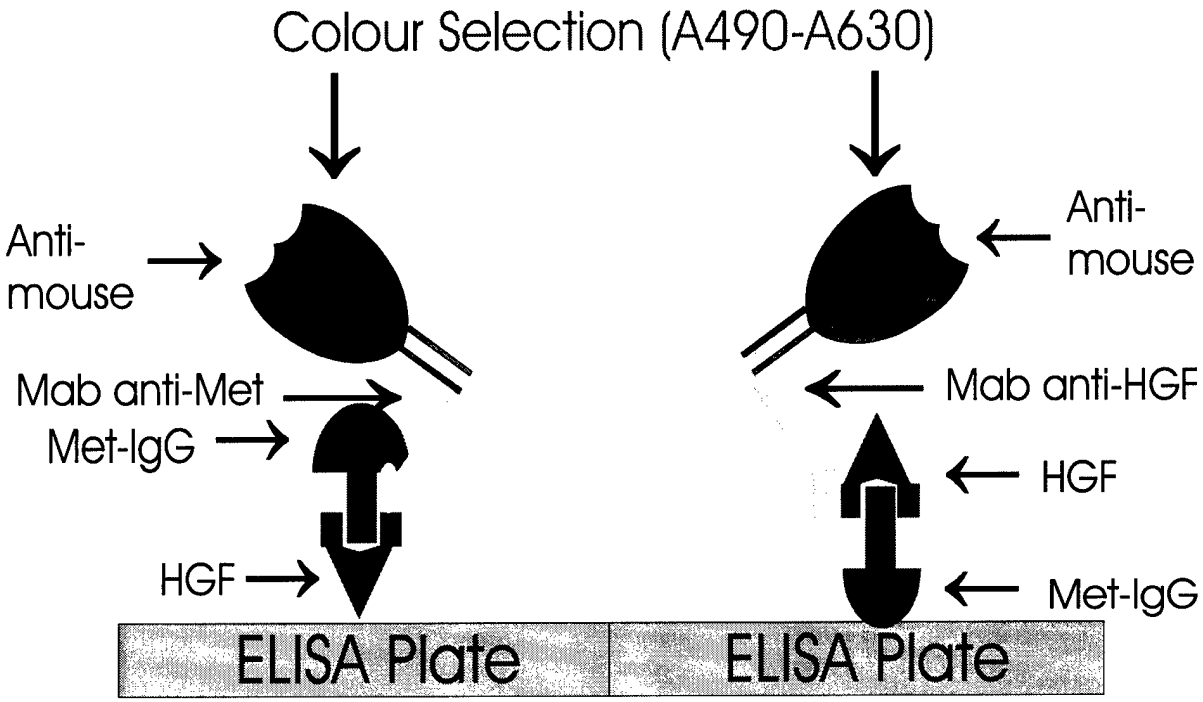

Figure 3. Detection of HGF or Met-IgG capture on an ELISA plate: An ELISA plate was coated with 50 ng of HGF (left) or 1000 ng of Met-IgG (right) overnight at 4°C. The plate was then washed 3x with PBS-Tween 20 (0.1%), followed by incubation with Met-IgG (left) or HGF (right) for 2 hours at room temperature. Similar washing was performed, followed by incubation with monoclonal anti-Met (left) or monoclonal anti-HGF (right) IgG for 2 hours at room temperature. Again, the plate was washed and then incubated with donkey anti-mouse conjugated to horse radish peroxidase (HRP) for 1 h at room temperature. Binding was detected by reaction with O-phenylenediamine and subsequent colour selection at A490 and A630.

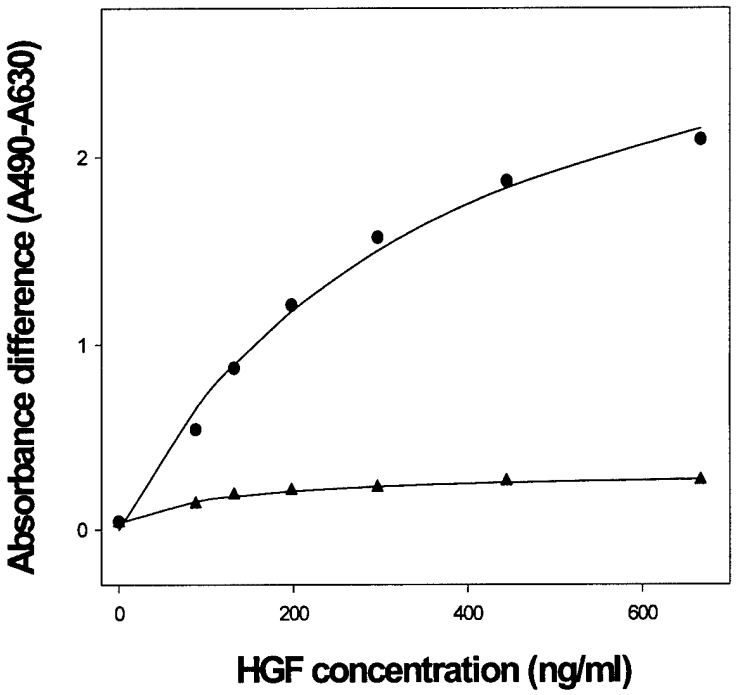

Figure 4. Detection of HGF binding to immobilized Met-IgG: HGF bound to immobilized Met-IgG (represented by right side of Figure 3), but showed very little binding to the immobilized human IgG control.

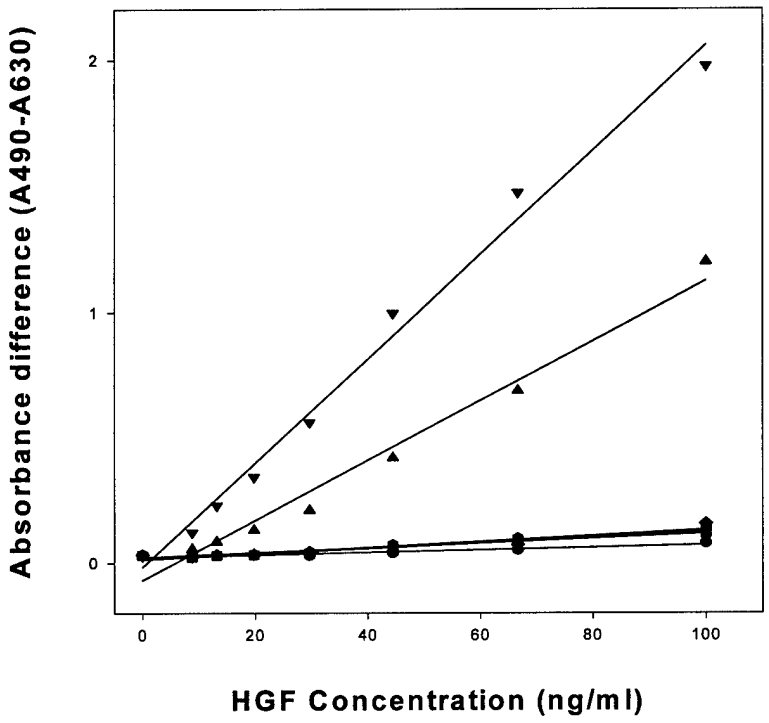

Figure 5. Effect of Cu^{2+} on HGF binding to immobilized Met-IgG: HGF bound to immobilized Met-IgG in the absence of Cu^{2+} (inverted triangle). High concentrations of Cu^{2+} (20 mM, triangle; 10 mM, square; 5 mM, circle; and 2.5 mM, diamond) abolished HGF binding to Met-IgG. A lower concentration of Cu^{2+} (1 mM, triangle) caused a partial inhibition of HGF binding to Met-IgG.

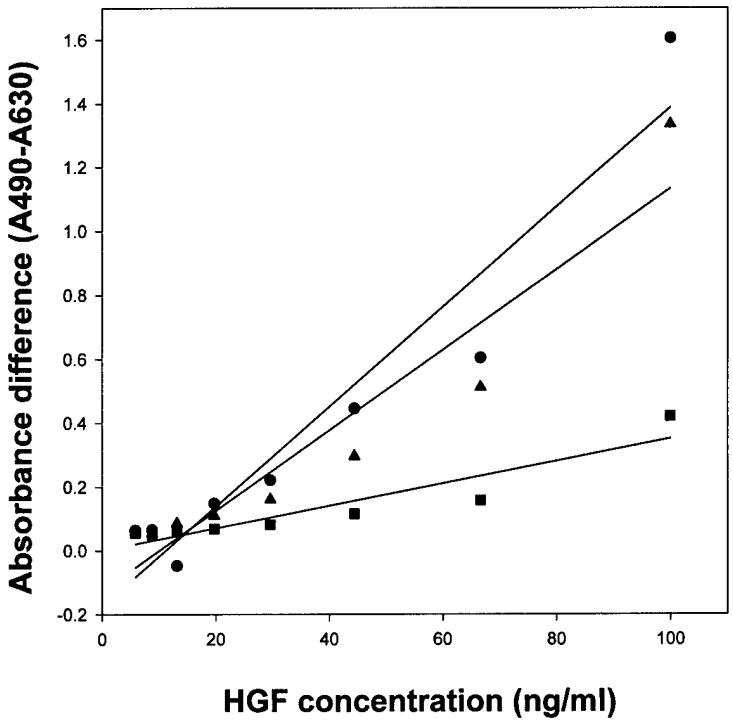

Figure 6. Effect of time of addition of Cu^{2+} on HGF binding to immobilized Met-IgG: HGF bound to immobilized Met-IgG in the absence of Cu^{2+} (circle). Co-incubation of HGF with Cu^{2+} (0.1 M, square) decreased binding of HGF to immobilized Met-IgG. In contrast, pre-incubation of HGF with immobilized Met-IgG and subsequent incubation with Cu^{2+} (0.1 M, triangle) showed very little change in HGF binding to immobilized Met-IgG.

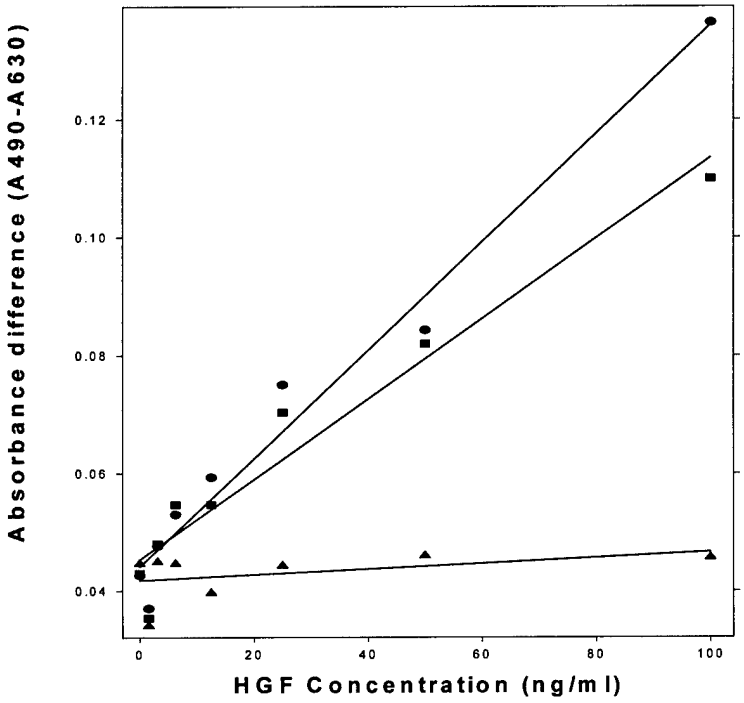

Figure 7. Effect of the lysine analogue, ε-ACA, on inhibition by Cu^{2+} of HGF binding to immobilized Met-IgG: HGF bound to immobilized Met-IgG (circle). Co-incubation of HGF with Cu^{2+} (0.1 M, triangle) decreased HGF binding to immobilized Met-IgG, while co-incubation of HGF with Cu^{2+} and ε-ACA (0.1 M, square) showed very little change in HGF binding to immobilized Met-IgG.

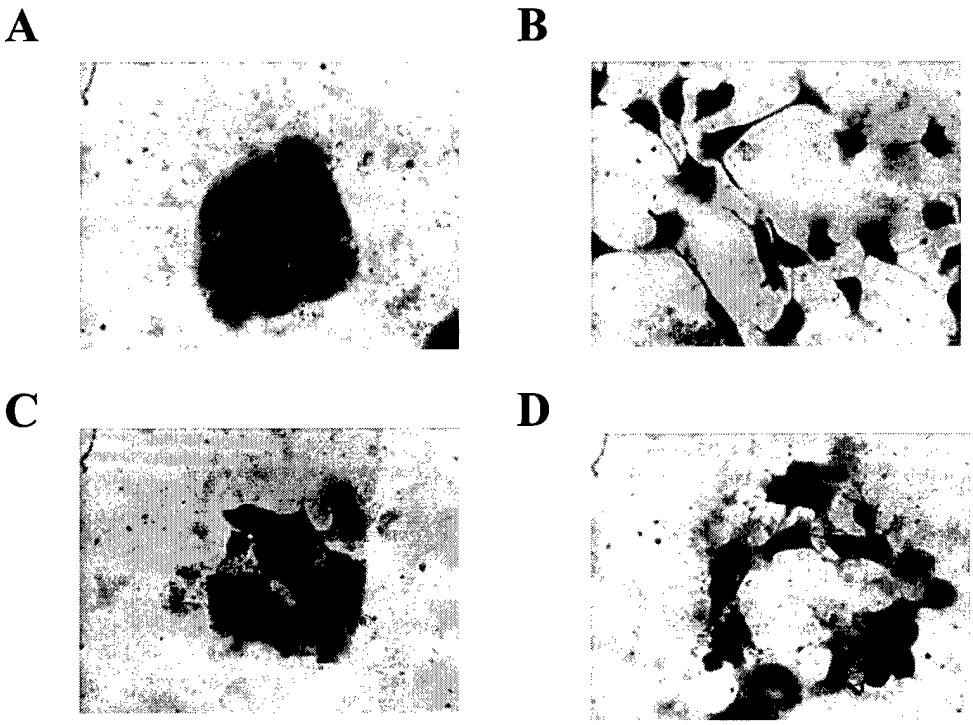

Figure 8. Effect of Cu^{2+} on HGF-induced scattering of MDCK cells: MDCK cells do not show scatter activity when in the presence of DMEM alone (A), while addition of HGF (20 ng) induced scatter (B). Addition of Cu^{2+} (500 μM) inhibited HGF-induced scatter (D), while Cu^{2+} alone (500 μM) had no scattering effect (C). Cells were stained with either hematoxylin (150x magnification) or DAPI (data not shown). No change in nuclear morphology of cells was detected.

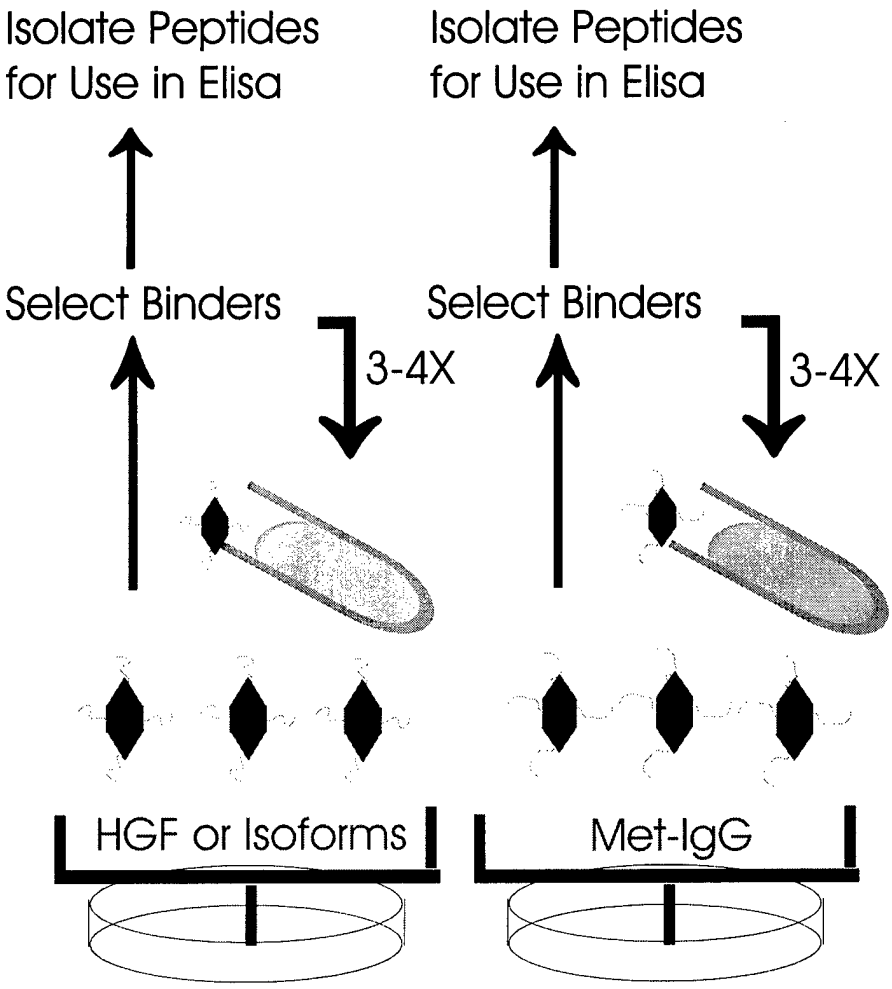

Figure 9. Schematic representation of phage display strategy: Bacteriophage expressing decapeptide or hexapeptide sequences on their protein coat are amplified using K91Kan (kanamycin resistant) bacteria. Phage are purified and incubated on plates coated with either HGF or isoforms, or plates coated with Met-IgG. Binders are selected and amplified using K91Kan bacteria again. This selection process is repeated 2 more times. Peptide sequences determined to be positive binders are then screened in an ELISA binding assay.

Appendices

1) Revised Statement of Work
2) Assistance Agreement
3) Abstract: CBCRI Grant 1999-2001.

Revised Statement of Work

PI: Theodore G. Wright

Title of Proposal: Identification of Hepatocyte Growth Factor autocrine loops in breast carcinomas: possible target for therapeutic intervention

Objective 1: To identify and assess the structural properties of native HGF, HGF degradation products and isoforms secreted by breast carcinoma cell lines and tissues; then to further determine the effects of putative HGF degradation products and isoforms on Met activation and function in breast epithelial and carcinoma cells.

A) Analysis of HGF degradation products:

- Preliminary screening of conditioned medium shows putative degradation products of HGF secreted by one breast carcinoma cell line. Therefore, screen various breast epithelial and carcinoma cell lines and tumor tissues that express endogenous HGF for degradation products of HGF (mo.4-5).
- Co-incubation of recombinant HGF with the above cell line causes increased intensity of HGF degradation products. Therefore, incubate recombinant HGF with the above breast epithelial and carcinoma cell lines, and determine the effect on the structural features of recombinant HGF. Assess the type of proteases involved using protease inhibitors (mo.1-3).
- purify the putative HGF degradation products by column chromatography and determine amino acid sequences and structure (mo.6-8).
- perform preliminary analysis of the effect of HGF degradation products on Met activation and function (e.g. cell scattering) (mo.9-10).

B) Analysis of putative HGF isoforms:

- extract total RNA from breast carcinoma cell lines and tumor tissues expressing putative HGF isoforms (mo.11-12).
- design primers and synthesize mRNA constructs corresponding to putative HGF isoforms (14-16).
- assess mRNA expression of native HGF and putative isoforms utilizing RT-PCR (mo.12-13).
- express recombinant HGF isoforms in 293 cells and purify (mo.17-20).
- analyze the effects of purified HGF isoforms on Met phosphorylation and function in breast epithelial and carcinoma cells, and compare with, full-length HGF, and two naturally occurring truncated isoforms of HGF (NK1 and NK2) (mo.18-21).

Objective 2: **to design high affinity peptides that either inhibit or promote the interaction between HGF and Met, and to assess the effect of these peptides on HGF/Met function.**

A) Design of high affinity peptides:

- develop screening assays for binding of HGF to Met that can be utilized for binding competition assays. Perform ELISA with immobilized antibodies to HGF or Met to capture the Met-HGF complex, which can then be detected by antibodies to Met or HGF (i.e. opposite to the captured substrate) (mo.1-3).
- design and construct a Met-binding peptide as a positive control (mo.4-6).
- amplify phage displaying positive control and random libraries. Confirm the diversity of random libraries (mo.7-9).
- immobilize control (α-chain with deleted K1 domain, and nonimmune IgG) and target proteins (Met-IgG and NK2) on separate polystyrene dishes (mo.10).
- perform the first stage of phage selection on random libraries and a positive control library to remove phage that bind epitopes not associated with the K1 domain of HGF, or the HGF-binding domain of Met (mo.11-12).
- elute desired binding phage, amplify and re-select (3-4 times) (mo.13-16).
- perform partial DNA sequencing on approximately 100 clones from each target selection (Met-IgG and NK1K2) (mo.17).
- determine relative binding affinities using the ELISA screening assay (mo.18-20)
- based on the deduced amino acid sequences and affinities, select 2 to 3 clones from each group for peptide synthesis (mo.21).
- use designed peptides in ELISA competition assays (and perhaps solution phase assays) with full-length HGF, NK1, NK2, and previously purified degradation products of HGF (mo.21-25)
- use these competition assays and computer modeling to predict docking sites to the corresponding target molecules, and types of interactions involved (mo.26-28).

B) Functional analysis of HGF/Met-binding peptides:

- analyze the effects of putative HGF/Met-binding peptides on tyrosine-phosphorylation of Met in breast epithelial and carcinoma cells using designed peptides, alone and in combination, with full-length HGF.
- compare with the effects of NK1 and NK2 isoforms and purified degradation products of HGF (see Objective 1A above) (mo.29-31).
- test peptides for specific binding to tissue sections and Met-activated/HGF-induced cellular functions (survival, growth, motility and invasion) (mo.32-36).
- based on these findings, choose candidate peptides for testing therapeutic intervention in future *in vivo* studies.

Queen's UNIVERSITY

CANCER RESEARCH LABORATORIES

Botterell Hall, Third Floor
Queen's University
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Tel 613 533-6567
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April 11, 2001

Sheri Labella
U.S. Army Medical Research Acquisition Activity
AttnMCMR-AAA-A
820 Chandler St.
Fort Detrick Maryland 21702-5014

Dear Sheri,

Re: Predoctoral Studentship award #DAM17-99-1-9360

This letter concerns Predoctoral Studentship award #DAM17-99-1-9360, entitled ***"Identification of hepatocyte growth factor autocrine loops in breast carcinomas: Possible targets for therapeutic intervention"***. Our Administrator, Mr. John Singleton, has previously contacted you concerning this award.

As you know the previous PI of this award, Jin Gui, withdrew from the PhD programme in May 2000 for academic and personal reasons. I have now recruited a new student, Ted Wright, to continue with this project. I would like to recommend Mr. Wright as the new PI for this award.

Mr. Wright recently completed his MSc degree in Biochemistry, under the supervision of Dr. Marlys Koschinsky on the role of kringle domains in the assembly of lipoprotein(a). His formal training in protein biochemistry and his knowledge of kringle domains provides an ideal background for this project. In addition, he has experience with site-specific mutagenesis, PCR, DNA sequencing, plasmid ligations, protein isolation, affinity chromatography and analysis of covalent and non-covalent binding interactions between plasma and recombinant proteins, as well as SDS-PAGE and tissue culture. He maintained an overall B+ average in five graduate courses in biochemistry, and made an excellent presentation of his thesis with only minor corrections required. He has one publication, one Abstract submitted, and one manuscript in preparation based on his previous work. A copy of his CV is attached for further information.

The effect of changing PIs on the nature of the project will be minimal. Moreover, the addition of Mr. Wright to our team brings new strengths to the project in the area of protein biochemistry.

.../2

His new supervisory committee members will include my collaborator, Dr. Zonchao Jia (Department of Biochemistry) who is a protein structure biochemist, and Dr. Greg Ross (Department of Pharmacology and Physiology) who is an expert in growth factor receptor inhibitor designs. Now that Mr. Wright is the PI of this project, I expect that the project will progress in a timely manner.

All safety and environmental requirements for this programme have been met, according to Queen's University regulations.

An update of the Statement of work required for the project is attached.

I wish to thank the USAMRMC for their flexibility in allowing me to propose a new PI for this project.

Sincerely,

Bruce Elliott

Bruce Elliott, PhD.
Professor, Department of Pathology
Cancer Research Laboratories
Ph. 613-533-2825
Fax. 613-533-6830
E-mail: elliottb@post.queensu.ca

BEE: jec
Encls.

ASSISTANCE AGREEMENT

AWARD TYPE: [√] GRANT (31 USC 6304) [ ] COOPERATIVE AGREEMENT (31 USC 6305) [ ] ER TRANSACTION (10 USC 2371)_____			
AWARD NO: DAMD17-99-1-9360, Modification P00002	EFFECTIVE DATE See Grants Officer Signature Date Below	AWARD AMOUNT $44,655.00	Page 1 of 2 Sherry L. LaBella 301-619-2806 fax 301-619-3166

PROJECT TITLE: "Identification of Hepatocyte Growth Factor Autocrine Loops in Breast Carcinomas: Possible Target for Therapeutic Intervention"

CFDA 12.420

PERFORMANCE PERIOD: 1 Jul 1999 - 1 Aug 2003 (Research to be completed by 1 Jul 2003)	PRINCIPAL INVESTIGATOR: Mr. Theodore G. Wright
AWARDED AND ADMINISTERED BY: U.S. Army Medical Research Acquisition Activity ATTN: MCMR-AAA-A 820 Chandler St. Fort Detrick Maryland 21702-5014	PAYMENTS WILL BE MADE BY: EFT:T Army Vendor Pay DFAS-SA/FPAC Phone: 888-478-5636 500 McCullough Avenue San Antonio Texas 78215-2100 *(SEE PARAGRAPH TITLED "PAYMENTS" FOR INSTRUCTIONS)*
DUNS No: 249920760 TIN No:	
AWARDED TO: Queen's University Faculty of Medicine Room 237, Botterell Hall Kingston, Ontario, Canada K7L 3N6	REMIT PAYMENT TO: Queen's University Office of Research Services Room 301 Fleming Hall, Jemmett Wing Kingston, Ontario, K7L 3N6, Canada

ACCOUNTING AND APPROPRIATION DATA:
N/A

SCOPE OF WORK:

BCRP - Predoctoral Training Award MENTOR: Dr. Bruce Elliott

a. The purpose of this modification is as follows:

(1) The Principal Investigator is hereby changed from Jin Gui to Mr. Theodore G. Wright.

(2) The Revised Statement of Work dated 17 May 2001 is hereby incorporated by reference.

(3) The original Mentor has returned from sabbatical; therefore, the mentor is hereby changed from Dr. Peter Greer to Dr. Bruce Elliott.

(4) Paragraph 23, USE OF HUMAN ANATOMICAL SUBSTANCES," is hereby deleted and replaced with the following paragraph 23:

RECIPIENT		GRANTS OFFICER	
ACCEPTED BY:		UNITED STATES OF AMERICA	
SIGNATURE		SIGNATURE	
NAME AND TITLE	DATE	NAME AND TITLE PATRICIA A. EVANS GRANTS OFFICER	DATE

23. USE OF HUMAN ANATOMICAL SUBSTANCES (NOV 2000) (USAMRAA)

a. The recipient, or its subrecipients, are authorized to conduct research under this award involving human anatomical substances for the following protocols:

Protocol Entitled "Identification of Hepatocyte Growth Factor Autocrine Loops in Breast Carcinomas: Possible Targets for Therapeutic Intervention," Submitted by Mr. Theodore G. Wright, Queen's University, HSRRB Log No. A-8681, Proposal No. BC980997, Grant No. DAMD17-99-1-9360

Protocols not identified are not approved.

b. Any anatomical substance (organs, tissues, or tissue fluids) linked by identifiers to a particular person and used for research under this award shall be donated for the purpose of research or investigation. The donor shall be the person from whom the substance is removed or, in the event of death or legal disability of the person from whom the substance is removed, the next of kin or legal representative of such person. Donation shall be made by written consent and shall relinquish all ownership and/or rights to the substance. All human anatomical substances used in research under this award shall be lawfully acquired. It should be noted that a general autopsy consent form or a consent to perform surgery, in and of themselves, may not be adequate. If excised or autopsy tissue is to be used, the protocol shall include a copy of the consent form used to obtain the tissue.

b. All other terms and conditions remain unchanged.

TOTAL GOVERNMENT FUNDS OBLIGATED: $44,655.00

CBCRI Grant (1999-01)

10. DETAILED SCIENTIFIC ABSTRACT/RÉSUMÉ SCIENTIFIQUE DÉTAILLÉ

Responses must be limited to one-half (½) page. Refer to the *1999 IDEA Grant Application Guide* for specific instructions regarding the format to be used for this section./Les réponses doivent être limitées à une demi-page. Consultez le *Guide de demande de subvention IDEA 1999* pour obtenir les directives spécifiques ayant trait au format à utiliser pour cette section.

Elliott, Bruce E. (PI)[1]
Cancer Research Labs. and Dept. Pathology, Queen's University

Title of Project: Development of peptide antagonists to hepatocyte growth factor as a novel approach to therapeutic intervention in breast cancer.

Keywords/Technical Terms: HGF; Met; peptides antagonists; cell survival; growth; treatment; breast cancer metastasis

Increased expression of HGF and its receptor, Met, have been identified as possible independent predictors of poor survival in breast cancer patients. We and others have shown that HGF is a potent stimulator of anchorage-independent survival, growth, tumorigenesis and metastasis of carcinoma cells. Together, these findings suggest that increased paracrine and autocrine activation of Met in carcinoma cells may be one important step in the development of invasive human breast cancer. Strategies to inhibit HGF/Met function, particularly HGF autocrine loops, may therefore provide novel therapeutic approaches for the treatment of invasive breast cancer. The recent advent of phage display technology has allowed the rapid identification of high affinity peptide antagonists that effectively block ligand-receptor binding and function. ***Our hypothesis is that invasive breast cancer can be targeted by HGF-binding, or Met-binding, oligomeric peptides that inhibit activation of Met and HGF-dependent survival, growth and metastasis of carcinoma cells.*** **The Objectives** of this proposal are:

I) To isolate from random phage display libraries high affinity peptide antagonists that block the interaction between HGF and Met ; and

II) To test the effect of peptide antagonists to HGF on Met activation and HGF-dependent survival, growth, and metastasis of breast carcinoma cells.

This project will identify and characterize potential novel peptide antagonists of HGF function in breast carcinoma cells. Lead peptides could then provide the basis for future study of the HGF binding site of Met and to develop more efficient inhibitor designs. High affinity peptide antagonists to HGF may be particularly useful in the disruption of HGF/Met autocrine loops, and may lead to the design of better procedures for diagnosis and treatment of invasive breast cancer.

11. FOCUS ON INNOVATIVENESS AND NOVEL APPROACH
ACCENT SUR L'INNOVATION ET L'ORIGINALITÉ DE L'APPROCHE

Responses must be limited to one-half (½) page. Explain why the project is innovative and novel. Explain why the project is not appropriate as a "feasibility grant application" to the next regular competition. Refer to *the 1999 IDEA Grant Application Guide*./Les réponses doivent être limitées à une demi-page. Expliquez pourquoi le projet est innovateur et original. Expliquez pourquoi le projet n'est pas approprié pour soumission dans le cadre du prochain concours habituel de "demande de subvention de faisabilité". Consultez le *Guide de demande de subvention IDEA 1999.*

The innovative aspect of this proposal is the identification of novel peptide antagonists that block interaction of hepatocyte growth factor (HGF) with its receptor Met, and inhibit HGF-induced tumorigenic and metastatic properties of breast carcinoma cells. The use of phage display allows the rapid identification of short peptides which bind with high affinity to certain growth factors or their receptors, block binding of the growth factor to its receptor, and effectively inhibit growth factor function. Previous approaches involving anti-HGF neutralizing antibodies or antisense technology may be insufficient to inhibit activation of Met *in vivo*, particularly in carcinoma cells producing both HGF and Met. In contrast, peptide antagonists isolated from random peptide libraries could have higher affinity for the receptor than the corresponding native peptide, and would therefore exhibit more efficient antagonistic activity. In addition, some antagonists of Met may act by directly inhibiting Met function. ***Peptide antagonists to HGF and Met have not been described before, and would provide a new approach that would be useful in the generation of anti-cancer agents.***

The current IDEA proposal is a high risk-high gain project that will allow the rapid screening for putative peptide antagonists of HGF and Met. Lead peptides identified in this IDEA project would then provide the basis to study the HGF binding site of Met and to develop more efficient inhibitor designs. The latter project would form the basis for a "feasibility" study or full investigation in a future competition.